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A rapid and efficient method for the screening of acid phosphatase 1 in young tomato seedlings, and for the identification of root-knot nematode species using miniaturized polyacrylamide gel electrophoresis

A relatively rapid and highly sensitive miniaturized polyacrylamide gel electrophoresis technique is described for the analysis of certain isozymes from single cotyledons of tomato seedlings and from single females of the root-knot nematode (*Meloidogyne* spp.). Homogenates from single tomato cotyledons (7, 14, 21, and 28 days old) were electrophoresed and stained for acid phosphatase 1 (*Aps 1*) activity. Cotyledons from plants of all the above age groups showed good *Aps 1* activity. Nondestructive screening for tomato *Aps 1* is therefore feasible, using very small samples, from as young as 7-day-old tomato seedlings. This could be of important use in expediting root-knot nematode resistance (based on the *Aps 1*-linked resistance gene *Mi*) screening for breeding programs, or F_1 testing for seed production purposes. In addition, the mini-polyacrylamide gel electrophoresis technique was useful for determination of the *Aps 1* allelic contribution to the total enzyme activity. The system was also used to detect malate dehydrogenase and esterase isozyme activity from single adult females of the four common root-knot nematodes, *Meloidogyne arenaria, M. hapla, M. incognita*, and *M. javanica*, with equally good results, enabling species discrimination.

1 Introduction

Isozyme analysis is an important tool in tomato plant breeding. Tagging useful traits [1, 2], gene mapping [3, 4], transfer of quantitative traits [5], and selection of interspecific hybrids [6] are good examples. Rick and Tanksley [7] reviewed the importance and utilization of isozymes in tomato breeding programs. Rick and Fobes [8] found a slow-moving variant allele of acid phosphatase 1 (*Aps* $1^{1/1}$) that in most cases was observed to segregate together with resistance to root-knot nematodes (RKN), *Meloidogyne* spp., conferred by the single dominant gene *Mi*. Further genetic studies demonstrated a tight linkage between the *Mi* and *Aps 1* loci, with the dominant *Mi* and *Aps* $1^{1/1}$ alleles associated [9]. This finding was an important improvement for tomato breeding programs because resistance to RKN could be screened by detecting the presence of the *Aps* $1^{1/1}$ allele. Most breeding programs use starch gel electrophoresis (SGE) for this purpose [7], which has the advantage of being less expensive than standard polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), and cellulose acetate electrophoresis (CAE). However, SGE demands much more time, and banding resolution and sensitivity are not as good as with some of the other techniques. The requirement of a relatively large amount of plant tissue sample to detect protein activity is another undesirable characteristic of SGE. In tomato, for example, if the plant is required to be grown to maturity, 6-to-8-week-old seedlings are necessary to provide enough leaf material for the detection of *Aps 1* (based on personal experience). CAE presents similar problems: despite better resolution and a faster procedure than SGE, cellulose acetate sheets are expensive and the technique also requires relatively large amounts of plant extract in order to detect enzymatic activity.

Although SGE and CAE have been used for the study of nematode isozymes, these techniques do not work well with all isozyme systems [10, 11]. When RKN extracts are used, at least 10 young females are required to detect enzymatic activity with SGE [10] and there is a similar requirement for CAE (personal experience).

A polyacrylamide gel electrophoresis technique is described which utilizes a mini-gel system that has the following advantages: (i) it is easy to operate and relatively inexpensive (the basic equipment cost is about $500), (ii) electrophoresis can be done on the bench at room temperature, and (iii) it is highly sensitive, allowing work with very small amounts of plant tissue and with single RKN females. The need for only a small amount of plant tissue can be particularly important in tomato breeding programs because screening for plants bearing the *Aps 1* variant allele can be carried out with a plant homogenate of a single 7-day-old cotyledon. This technique could expedite the screening of tomato lines resistant to RKN, the resultant savings in time and labor being valuable aspects of breeding programs. In addition, this technique is useful for the specific identification of RKN, with the advantage over more sophisticated systems like the Phast-gel [12] of being more versatile and economical without sacrificing quality. A comparison is made of the amount of *Aps 1* protein detected in a single cotyledon from 7-, 14-, 21-, and 28-day-old tomato seedlings and the allelic contribution of each to the enzymatic activity of *Aps 1*.

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Aps, acid phosphatase (EC 3.1.3.2); **%C**, crosslinking agent (g) × 100/%T; **EST**, esterase (3.1.1.1); **MDH**, malate dehydrogenase (1.1.1.37); **PAGE**, polyacrylamide gel electrophoresis; R_f, relative migration, band migration (mm)/anodal front line (mm) × 100; **RKN**, root-knot nematode; **%T**, acrylamide (g) + Bis (g)/100 mL

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2 Materials and methods

2.1 Plant material

Seedlings of tomato cultivars VFN, Carmen, and Bonny Best, characterized as being homozygous resistant, heterozygous resistant, and homozygous susceptible to RKN, respectively, were used. These cultivars were chosen on the basis of their *Aps 1* phenotypes, which are shown in Fig. 1. Plants were grown in speedling trays containing 196 wells filled with vermiculite, in the greenhouse, with a day/night temperature regime of 25 ± 5 °C. Seeds of the three tomato cultivars were planted at 7-day intervals for 28 days and the seedlings were fertilized with Osmocote (Sierra Chemical Co., Corvallis, OR). The weight and length of each cotyledon were recorded for each treatment and a single cotyledon was taken for protein extraction.

	VFN (*1/1*)*	Carmen (*1/+*)	Bonny Best (+/+)
(−)	−	−	
↓		−	
(+)		−	−

* Allelic notation.

Figure 1. *Aps 1* phenotypes of resistant and susceptible homozygous and resistant heterozygous tomato genotypes used in this study.

2.2 PAGE procedure for *Aps 1* protein

A miniature vertical slab gel electrophoresis unit, Mighty Small II model SE 250 (Hoefer Scientific Instruments, 654 Minnesota St., PO Box 77387, San Francisco, CA) was used. The system has slabs, 75 × 80 mm, that can be used with different spacers to obtain a gel thickness that can be varied from 0.50 to 1.5 mm. A casting chamber is supplied that enables the preparation of many gels, which, once polymerized, may be wrapped in a plastic wrap and stored in a refrigerator at 4 °C for up to 10 days.

2.3 Preparation of the gel

The resolving gel was 1.5 mm thick and contained 8% T and 2.7% C_{Bis} (*N,N'*-methylenebisacrylamide); 0.38 M Tris-HCl, pH 8.9; 0.018% w/v ammonium persulfate; and 0.029% v/v *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 4% T and 20% C_{Bis}; 0.06 M Tris-HCl, pH 6.7; 0.058% v/v TEMED, 0.0005% w/v riboflavin; and 5% w/v sucrose. Tank buffer was 0.05 M Tris, 0.38 M glycine, pH 8.3.

2.4 Sample preparation

Protein extraction from single cotyledon samples (7-, 14-, 21-, and 28-day-old seedlings) was performed according to Suurs *et al.* [13]. Each single cotyledon was ground in a 1.5 mL microcentrifuge tube in 50 µL of extracting buffer containing 10 mL of 0.05 M Tris, 2 mL of glycerol and 100 µL of 2-mercaptoethanol. The extracts were centrifuged at 14000 *g* for 3 min and then 10 µL of sample were loaded directly into a gel well. The central well, labeled P (Fig. 2), was loaded with 10 µL of a solution containing pure *Aps 1*. The amount of protein in this sample was determined by the Bradford method [14]. A calibration curve with bovine serum albumin (BSA) was determined in parallel with the unknown sample of *Aps 1* in order to estimate the sample protein concentration using a Beckman PBU 70 spectrophotometer.

2.5 Running conditions

Polyacrylamide gel electrophoresis was carried out at 80 V for the first 30 min and then at 200 V constant voltage until the front line had reached the bottom buffer chamber, after about 30 min.

2.6 *Aps 1* staining procedure

The gel was incubated with stain solution overnight in an incubator at 32 °C [15]. Composition of the stain solution was 10 mL of 0.5 M Na acetate, pH 4.5, 1.5 mL of 0.05 M $MgCl_2.6H_2O$, 125 mg of Fast Black K salt, 2.5 mL of 1% β-naphthyl phosphate (in 50% acetone) and distilled water up to 100 mL. After staining, the gel was fixed in 7% glacial acetic acid solution, and then dried by wrapping it between two sheets of BioGelWrap (BioDesign Inc. of New York, P.O.Box 1050, Carmel, NY 10512). The dried gel is completely transparent and the stain colors are well preserved. Photographs were taken directly of the dried gel lying on a light box (Fig. 2). The position of isozyme bands was measured and the R_f values were calculated. In order to calculate the amount of protein detected for each cotyledon age, the dried gel was scanned with an LKB Ultrascan XL densitometer. An LKB 2210 recorder was used and the peak recorded for each isozyme band on the gel was defined, cut and weighed and the amount of protein in those bands was estimated indirectly by comparison with the peak obtained for the sample with the known concentration of *Aps 1*.

2.7 Nematode material

Females of *Meloidogyne incognita, M. arenaria, M. javanica,* and *M. hapla* were cultured on tomato cv. Rutgers plants maintained in the greenhouse [16].

2.8 Nematode sample preparation

Single RKN females were taken from dissected roots and placed in microcentrifuge tubes containing 15 µL of cold extracting buffer (20 mL glycerol, 2 mL Triton X-100, and distilled water up to 100 mL). The female in buffer was macerated inside the centrifuge tube with a pestle and centrifuged for 4 min at 14000 rpm. The sample was stored at −80 °C.

2.9 Gel composition

Resolving gel composition was acrylamide 7% T, 2.7% C_{Bis}, 0.19 M Tris-HCl, pH 8.6. The stacking gel composition was the same as for tomato *Aps 1*. Tank buffer was 0.005 M Tris and 0.038 M glycine, pH 8.3. The gel was loaded with 10 µL of the nematode homogenate per well. One drop of 1% Bromophenol Blue was added to the upper buffer chamber, mixed with the buffer and used as a track dye.

2.10 Running conditions

Electrophoresis of nematode extracts was performed as described above (Section 2.5) and stopped when the Bromophenol Blue line reached the bottom buffer chamber.

2.11 Malate dehydrogenase and esterase staining

Gels were stained first for malate dehydrogenase (MDH) for 5 min, rinsed with distilled water for 1 min and then stained for esterase (EST) for 1 h [12]. In both cases the gel was incubated at 32°C. Gels were then dried as described above. The composition of the stain solution for MDH was 0.1 M Tris, pH 7.1, 10.6 g/L sodium carbonate, 1.34 g/L L-malic acid, 0.5 g/L nicotinamide adenine dinucleotide, 0.3 g/L NitroBlue Tetrazolium and 0.02 g/L phenazine methosulfate. Stain solution for EST was 0.1 M potassium phosphate buffer, pH 7.2, 0.3 g/L ethylene diamine tetra acetic acid, 0.6 g/L Fast Blue RR salt and 0.4 g/L α-naphthylacetate (dissolved in 20 mL acetone). All stain solutions were filtered through a Whatman # 40 filter paper to remove insoluble material [17].

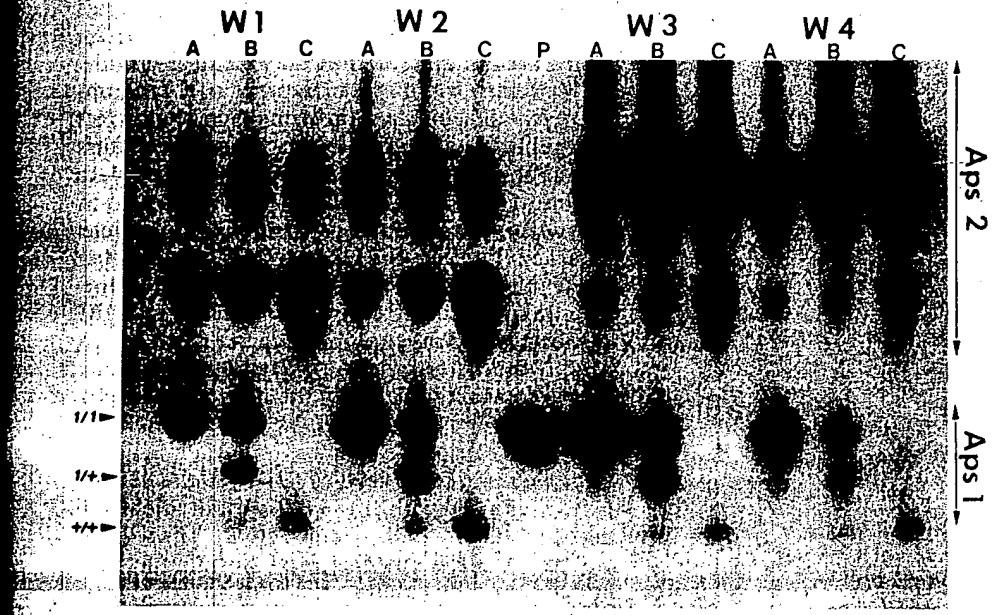

Figure 2. Acid phosphatase zymogram for *Aps 1* (chromosome 6) and *Aps 2* (chromosome 8, Rick [24]). W1, W2, W3, and W4: 7-, 14-, 21-, and 28-day-old plants, respectively. P: *Aps* $1^{1/1}$ pure protein. A, B, and C: tomato cultivars VFN, Carmen, and Bonny Best, respectively.

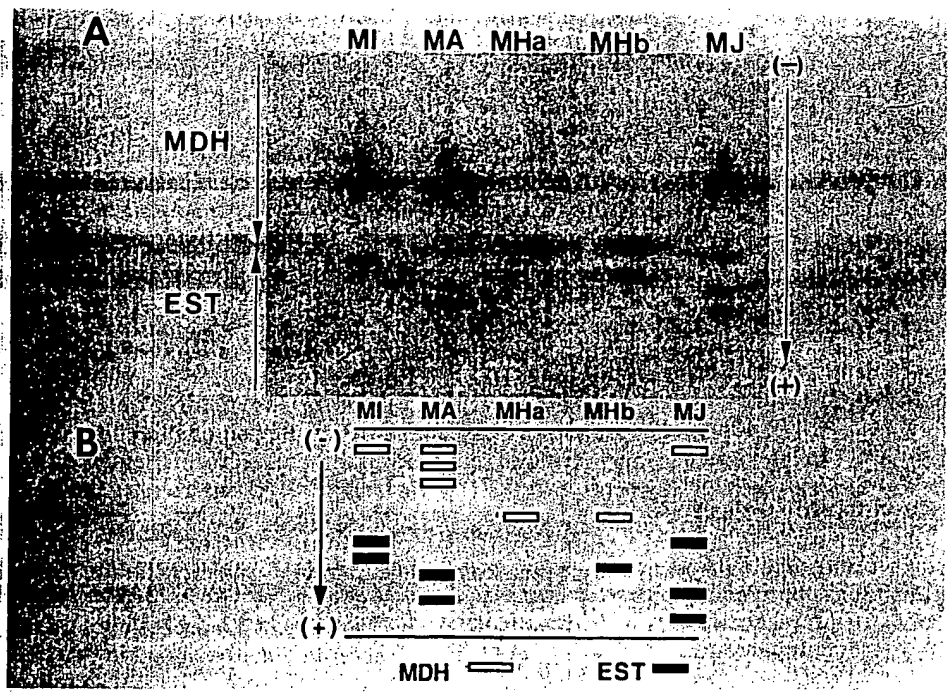

Figure 3. Photograph (A) and diagram (B) of MDH and EST phenotypes of single females of *Meloidogyne incognita* (MI), *M. arenaria* (MA), *M. hapla*, Angleterre isolate showing no EST activity (MHa), *M. hapla*, San Bernardino isolate with EST activity (MHb), and *M. javanica* (MJ).

3 Results

3.1 Enzymatic activity of *Aps 1*

The length and weight of single cotyledons (mean of five replicates) are given in Table 1 for each of the planting dates and tomato genotypes. Plants started to show the first true leaves 14 days after planting. The R_f values for tomato *Aps 1* obtained under these electrophoretic conditions were: 66.6 for the homomeric allele *1/1* associated with the resistant tomato genotype; 83.3 for the homomeric allele *+/+* of the susceptible tomato genotype (Fig. 2); and, for *Aps 1* of the heterozygous resistant genotype (*1/1*, *1/+*, and *+/+*), 66.6, 73.3, and 83.3, respectively (Fig. 2). The relative amount of *Aps* $1^{1/1}$ pure protein detected in the gel was 3.11 µg (Fig. 2, lane P). Table 2 shows the relative amount of *Aps* $1^{1/1}$ protein detected in a single cotyledon for each planting date and tomato genotype used in this study.

Table 1. Mean length (L) and weight (W) of single tomato cotyledons at different days from planting

Days after planting	VFN		Carmen		Bonny Best	
	L (mm)	W (mg)	L	W	L	W
7	14	7.6	12	4.8	15	7.7
14[a)]	20	15.0	18	11.1	20	12.7
21	23	17.0	19	11.0	25	16.8
28	27	24.0	25	18.3	25	18.6

a) Seedlings start to show first true leaves. Values are mean of 5 measurements.

Table 2. Amount of *Aps 1* in gel bands, expressed in µg of protein, from single cotyledons of tomato seedlings of different ages[a)]

Days after planting	VFN		Carmen		Bonny Best	*Aps 1* pure protein
	1/1	*1/1*	*1/+*	*+/+*	*+/+*	*1/1*
7	2.64	1.17	0.47	0.14	0.46	
14	3.88	1.12	0.83	0.17	0.81	3.11
21	5.18	1.77	0.90	0.11	0.39	
28	4.09	0.91	0.43	0.12	0.33	

a) Bands are shown in Fig. 2.

3.2 Activity of EST and MDH from single RKN females

Root-knot nematode MDH and EST zymograms for *Meloidogyne incognita*, *M. arenaria*, *M. hapla*, and *M. javanica* are shown in Fig. 3.

4 Discussion

In preliminary tests, although gels at 7% T and 2.7% C_{Bis} gave good resolution of tomato *Aps 1* bands (data not shown), the fast band corresponding to the *+/+* allele migrated almost together with the front line ($R_f = 100$). When %T was increased to 8%, the R_f value for the fast band (*+/+* allele) was 83.3 and sharpness and resolution were not affected (Fig. 2). A single 7-day-old tomato cotyledon with an average weight between 4.8–7.7 mg and a length between 12–15 mm (Table 1), showed good activity for *Aps 1* and *Aps 2* in the three cultivars tested (Fig. 2). Seedlings deprived of one cotyledon as early as 7 days old, did not show a noticeable difference in subsequent growth or vigor compared to unsampled seedlings of the same age.

These results are in contrast to those obtained with starch gel electrophoresis by Medina Filho and Tanksley [9], who observed that dry seeds, and seedlings up to 2 weeks old, including their corresponding basal stem and cotyledons, did not show good activity of *Aps 1*. The same authors found that 3-week-old tomato cotyledons also showed low activity and in some plants no *Aps 1* bands were detected. Detection of *Aps 1* activity from single 1-week-old cotyledons was also not good when cellulose acetate was used (personal experience). The results obtained in this study (Fig. 2) clearly demonstrate the sensitivity of the miniature vertical PAGE unit for the detection of *Aps 1* activity from very small plant samples.

Quality and resolution of *Aps 1* bands obtained from single cotyledons using mini-PAGE did not differ for the 4 cotyledon stages of development used in this study (Fig. 2). Estimates of the amount of protein in these samples (Table 2) indicated that the relative rate of synthesis of polypeptide coded by each allele of *Aps 1* in a single cotyledon, either in the homomeric or heteromeric form, improved during the first 21 days, and then decreased by 28 days; the electrophoretic pattern for *Aps 1* (Fig. 2; Table 2) suggests it is asymmetric. The contribution of the *Aps 1* wild-type allele *+/+* to the total activity is less than the variant allele *1/1*. *Aps 1* is a dimer [18, 19], and if the polypeptides coded by the two alleles make equal contributions to the enzyme activity and the subunits combine at random, the ratio of enzyme activity in the heterozygote (*+/1*) should be 1:2:1, with 50% of the total activity due to the homomeric form (*+/+* and *1/1*, the extreme bands of the heterozygote). However, the pattern observed here is asymmetric and independent of cotyledon age; the ratio of activity in the heterozygote was 2.5:1.25:0.25, with the *Aps 1* variant allele *1/1* contributing 62.5% of the total enzyme activity. The wild allele *Aps* $1^{+/+}$ contributed only 6.25% of the total activity, whereas the activity of the heteromeric form was 31.25% of the total. Harris and Hopkinson [17] pointed out that asymmetrical patterns could be a consequence of differences in stability or due to catalytic differences between polypeptide chains synthesized by two alleles.

The mini-gel technique described here is not only useful as a nondestructive method for early screening of tomato *Aps 1* associated with resistance to RKN, but it could also be useful for the study of allelic dosage effects in isozyme activity. Once dried, the gel maintains a clear background and the intensity of the bands allows an easy scanning of the gel by a densitometer. This is not feasible with starch gels or cellulose acetate membranes due to either the gel thickness or the opaqueness of the supporting medium.

Enzymatic activity for single females of RKN (Fig. 3) was strong for MDH and EST for the four species of *Meloidogyne* tested, and the R_f values are close to those obtained by other authors [12, 20]. These results demonstrated the high sensitivity and reliability of this technique for nematode extracts.

This mini-PAGE system performed with the Hoefer apparatus has been shown to be extremely sensitive for the detection of *Aps 1* phenotypes associated with root-knot nematode (*Meloidogyne* species) resistant or susceptible tomato genotypes. Only one 1-week-old tomato cotyledon is necessary to detect *Aps 1* activity, with an excellent resolution

which cannot be achieved with starch gels or cellulose acetate electrophoresis. The time required is relatively short (for tomato *Aps 1*, 60 min total run for 15 samples using a 1.5 mm thick slab, and the time can be reduced further if 0.5 or 0.75 mm thin slabs are used) compared with starch gels (3 h for 25 samples). Electrophoresis with the Hoefer apparatus takes longer than with the cellulose acetate technique described by Bolkan *et al.* [21] (15 min for 8 samples), but overall time is saved in its application for screening because only 7-day-old plant material is required. Seven-day-old seedlings have a small size, and thousands of seeds could be plated in Petri dishes and screened for nematode resistance by taking only a single cotyledon per plant, without destroying them. Time and reliable screening procedures are key factors in any breeding program. Rapid identification of undesirable inbred seeds in F_1 seed production programs could also make good use of this technique (see [22] for the use of ultrathin-layer isoelectric focusing of seed protein for this purpose). In addition, the Hoefer apparatus has given excellent results for the identification of RKN, and also it has been used to distinguish different geographical isolates of the false root-knot nematode *Nacobbus aberrans* (see [23]). Thus the mini-PAGE can be used as a powerful tool for diagnosis and nematode population dynamics studies.

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